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Adolescents' cortisol responses to awakening and social stress; Effects of gender, menstrual phase and oral contraceptives. The TRAILS study

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Summary Studies on the influence of sex hormones on cortisol responses to awakening and stress have mainly been conducted in adults, while reports on adolescents are scarce. We studied the effects of gender, menstrual cycle phase and oral contraceptive (OC) use on cortisol responses in a large sample of adolescents. Data come from TRAILS (TRacking Adolescents' Individual Lives Survey), a prospective population study of Dutch adolescents. This study uses data of 644 adolescents (age 15—17 years, 54.7% boys) who participated in a laboratory session including a performance-related social stress task (public speaking and mental arithmetic). Free cortisol levels were assessed by multiple saliva samples, both after awakening and during the laboratory session. No significant effects of gender and menstrual phase on cortisol responses to awakening were found, while girls using OC displayed a slightly blunted response ($F(1, 244) = 5.30$, $p = .02$). Cortisol responses to social stress were different for boys and free-cycling girls ($F(3, 494) = 9.73$, $p < .001$), and OC users and free-cycling girls ($F(3, 279) = 15.12$, $p < .001$). Unexpectedly, OC users showed no response at all but displayed linearly decreasing levels $F(1, 279) = 19.03$, $p < .001$) of cortisol during the social stress test. We found no effect of menstrual cycle phase on cortisol responses to social stress ($F(3, 157) = 0.58$, $p = .55$). The absence of a gender difference in the adolescents' cortisol awakening response found in this study is consistent with

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previous reports. Our results further suggest that adolescent OC users display slightly blunted cortisol responses after awakening, and that gender differences in cortisol responses to social stress during adolescence are comparable to those described for adult populations, that is, stronger responses in men than in women. Whereas previous work in adults suggested blunted stress responses in OC users compared to men and free-cycling women, adolescent OC users showed no cortisol response. Effects of type of OC could not be studied because of low numbers of OC that were only progestin based.

1. Introduction

Adolescence is an important period to study psycho-physiological responses to stress, because it is associated with increasing levels of sex hormones which modulate activation and feedback of one of the major stress systems of the body, the hypothalamus–pituitary–adrenal (HPA)-axis (Conrad et al., 2004). Nevertheless, studies concerning responses of the HPA-axis towards social stress have mainly been conducted in adult populations (Wüst et al., 2000; Kudielka and Kirschbaum, 2005; Kajantie and Phillips, 2006; Uhart et al., 2006). This is remarkable since animal studies indicate that adult HPA-axis responses to acute and chronic stress are different from those in adolescents, for instance, adolescent rats have been found to show prolonged HPA activation after exposure to stressful stimuli compared to adult rats (Romeo et al., 2006; McCormick and Mathews, 2007). Previous research on cortisol responses to social stress tasks suggests no differences between boys and girls before the onset of puberty (Buske-Kirschbaum et al., 1997; Tout et al., 1998). Increases in sex hormones are dissimilar for adolescent boys and girls, which may be one of the mechanisms for the profound gender difference in stress-related disorders that emerge in adolescence (Nolen-Hoeksema, 2001) such as depression (Angold et al., 1998).

During adolescence, estrogen and progesterone levels are increasing in girls, while testosterone levels rise in boys. In male rodents, stress responses are diminished by high levels of testosterone (Viau, 2002), but in humans the influence of testosterone on the stress response is not very clear. Animal studies further indicate that estrogens have complex regulatory effects on cortisol receptors, resulting in up and down regulation of stress responsiveness, depending on type of receptor, region of the brain where the receptors are located, and estrus cycle (Pfeiffer et al., 1991; Burgess and Handa, 1992; Handa et al., 1994). Estrogen promotes the synthesis of corticoid binding globulin (CBG) (Moore et al., 1978), which can bind up to 90% of free circulating cortisol (Siiteri et al., 1982). In this way, it can alter both activation and feedback of the HPA-axis during homeostasis, stress and pharmaceutical challenges (Jacobs et al., 1989; Kirschbaum et al., 1999; Kumsta et al., 2007). Progesterone can diminish the effectiveness of the HPA-axis feedback by binding to cortisol receptors (Svec, 1991) and increase the rate of dissociation of cortisol from such receptors (Rousseau et al., 1972). Several studies (reviewed by Kudielka and Kirschbaum, 2005) have shown that men display higher levels of cortisol than women in response to laboratory social stress tasks. This is contrary to findings regarding cortisol responses towards awakening, which rather seem to indicate that women have higher levels than men 30 min after awakening (Pruessner et al., 1997; Wüst et al., 2000), although this gender difference was not always replicated (Edwards et al., 2001). Because of the difference effects of male and female sex hormones on HPA function, adolescent boys and girls are also likely to display different cortisol responses to stress. Because of the cortisol-binding effects of estrogens, we expected lower cortisol responses in girls than in boys.

In girls, levels of sex hormones not only increase during adolescence, but also fluctuate during the menstrual cycle. In the follicular phase, the period before ovulation, estrogen levels are high and progesterone levels are low, while in the subsequent luteal phase progesterone levels are high while estrogens are lower than in the follicular phase (Fox, 1999). Differences in phase have not been associated with the cortisol response to awakening (Kudielka and Kirschbaum, 2003), but fluctuations in estrogen and progesterone levels may influence the response and feedback-loop of the HPA-axis during stress. Women in the luteal phase have shown higher cortisol responses than women in the follicular phase (Altemus et al., 1997; Kirschbaum et al., 1999; Wolf et al., 2001; Rohleder et al., 2003). This suggests a reduced sensitivity of the HPA feedback loop in periods when estrogen levels are high.

Adolescence is a period of sexual maturation, in which boys and girls start to engage in sexual activities and girls start using oral contraceptives (OC). Most of these OCs contain ethinylestradiol and a progestin that inhibit ovulation and reduce natural levels of estrogen and progesterone (Likis, 2002). Like natural estrogens, ethinylestradiol induces CBG synthesis (Wiegratz et al., 2003), resulting in an increasing level of CBG during the course of OC-intake. In concordance with this (Reinberg et al., 1996), found that OC users had lower mean salivary cortisol values than non-users during a 24-h cortisol assessment profile. With respect to the influences of OC on the cortisol response to awakening (CAR), Pruessner et al. (1997) report an attenuated CAR, but this was not replicated by Wüst et al. (2000). When confronted with a social stress task, OC users had lower saliva cortisol compared to free-cycling women in several studies (Kirschbaum et al., 1999; Rohleder et al., 2003). However, Brody (2002) reported no differences between OC users and non-users in cortisol responses to a social stress test. Hence, findings concerning the effects of OC on awakening and social stress are contradicting. This might be due to the duration of OC-intake; possibly, long-term OC use has a different effect on the response to stress than use for a shorter period of time.

Higher-order regulatory parts of the cortical brain are still maturing during adolescence (Gogtay et al., 2004). Corticol activities regulate activities of subcorticol regions such as the hypothalamus and the amygdala (Lewis and Todd, 2007), and thus emotional responses may be different in adolescents compared to adults. Sex hormones influence mood and coping with stress because of their interactions with receptors in these brain areas (Chrousos et al., 1998; Levine, 2002). It is

therefore highly relevant to study hormonal factors involved in the function of the HPA-axis in adolescence. This study concerns cortisol responses to awakening and to performance-related social stress in a large sample of adolescent boys and girls. We examined possible modulating effects of sex hormones by comparing boys and girls, and by examining effects of menstrual cycle phase and oral contraceptives.

2. Methods

2.1. Participants

The data were collected in a focus sample of TRAILS (TRacking Adolescents' Individual Lives Survey), a large prospective population study of Dutch adolescents with bi- or triennial measurements from age 11 to at least age 25. TRAILS participants were selected from five municipalities in the Northern part of the Netherlands. The three assessments waves finished so far ran from, respectively, March 2001 to July 2002 (T1), September 2003 to December 2004 (T2), and September 2005 to December 2007 (T3). At T1, 2230 children were enrolled in the study (response rate 76.0%, see De Winter et al., 2005) of whom 1816 (81.4%) participated at T3.

During T3, 744 adolescents were invited to perform a series of laboratory tasks (hereafter referred to as the experimental session) on top of the usual assessments, of whom 715 (96.1%) agreed to do so. Adolescents with a high risk of mental health problems had a greater chance of being selected for the experimental session. High risk was defined based on baseline temperament (high scores on frustration and fearfulness, low scores on effortful control), parental psychopathology (depression, anxiety, addiction, psychoses, or antisocial behavior), and environmental risk (living in a single-parent family). In total, 66.0% of the sample had at least one of the above-described risk factors. The remaining 34.0% were randomly selected from the total TRAILS sample. More detailed information on the selection procedure and response rates within each stratum can be obtained from the corresponding author. Adolescents with missing data on OC use or menstrual cycle phase were discarded, leaving a sample of 644 adolescents (mean age 16.13, S.D. = 0.59, 54.7% boys) for analysis.

2.2. Procedure

2.2.1. Experimental session

During the experimental session, participants' psycho-physiological responses (cardiovascular, cortisol, subjective arousal) to a variety of challenging conditions were recorded. These conditions included orthostatic stress (from supine to standing), a spatial orienting task, a gambling task, a startle reflex task, and a social stress test. The experimental protocol was approved by the Central Committee on Research Involving Human subjects (CCMO). The test assistants, 16 in total, received extensive training in order to optimize standardization of the experimental session.

The experimental sessions took place on weekdays, in sound-proof rooms with blinded windows at selected locations in the participants' residence town. The sessions lasted about 3 h and 15 min, and started between 08:00 h and 09:30 h (morning sessions, 49%) or between 01:00 h and 02:30 h (afternoon sessions, 51%). Although free salivary cortisol levels may be higher in the morning due to the circadian rhythm of cortisol production, morning and afternoon cortisol responses to social stress have been reported to be comparable (Kudielka et al., 2004). The participants were asked to collect two morning saliva samples on the day of the experimental session, one directly after waking up (Co1) (mean time of awakening = 07:39 h, S.D. = 1:10 h) and one 30 min later (Co2). They were instructed not to eat, brush their teeth, or engage in heavy exercise during this half hour, and to bring the tubes with them to the test location. In addition, we asked the participants to refrain from smoking and from using coffee, milk, chocolate, and other sugar-containing foods in the 2 h before the session. At the start of the session, the test assistant, blind to the participants' risk status, explained the procedure and administered a short checklist on current medication use (including OC), quality of sleep, and physical activity in the last 24 h, and attached the equipment for heart rate and blood pressure measurements. Next, participants filled out four computerized questionnaires, assessing life events in the past week, state and trait anxiety, mood states, and feelings and thoughts in the last month. The participants were asked to relax until 35 min after the start of the session. After this period of rest, heart rate and blood pressure were recorded for a period of 5 min, in which the participants had to sit still and were not allowed to speak. Afterwards, the first cortisol sample was collected (Ce1). Subsequently, the challenges (i.e., laboratory tasks) were administered in the before-mentioned order. Every task was followed by a short break, during which participants reported subjectively experienced arousal. The social stress test was the last challenge of the experimental session. Detailed information about this test is presented in the next paragraph. Following the social stress test, the participants were debriefed about the experiment and could relax for about 15 min, after which heart rate and blood pressure were recorded once more and anxiety and mood were assessed again.

2.2.2. The Groningen Social Stress Test (GSST)

The GSST is a standardized protocol, inspired by the Trier Social Stress Task (Kirschbaum et al., 1993) for the induction of moderate performance-related social stress. The GSST has been found to elicit significant changes in heart rate and in the HPA-system (Benschop et al., 1998; Van der Pompe et al., 1998). During the GSST, heart rate was recorded continuously. Blood pressure was not recorded so that the participants could move their hands freely to express themselves during the task. Participants were, on the spot, instructed to prepare a 6-min speech about themselves and their lives and deliver this speech in front of a video camera. They were told that their videotaped performance would be judged on content of speech as well as on use of voice and posture, and rank-ordered by a panel of peers after the experiment. The risk if being judged negatively by peers was included to induce threat of social rejection. Participants had to speak continuously for the whole period of 6 min. The test assistant watched the performance critically, without showing empathy or encouragement. After 6 min of speech, the participants were told that there was a problem with the computer and they had to sit still and be quiet. This interlude lasted 3 min, and was meant to asses cardiovascular recordings

without the disturbance of speech on respiration recordings. After the interlude, participants were instructed to subtract 17 repeatedly, starting with 13,278. This difficult task was meant to induce a sense of uncontrollability. Uncontrollability was further provoked by negative feedback by the test assistant, including remarks such as, ''No, wrong again, begin at 13,278'', ''Stop wiggling your hands'' or ''You are too slow, be as quick as you can, we are running out of schedule''. After 6 min of mental arithmetic, heart rate was recorded again during a 3-min period in which the participant was not allowed to speak. Directly after the GSST participants were ask to report their subjective arousal.

2.2.3. Saliva sampling during the GSST

HPA-axis responses towards the GSST were assessed by four cortisol samples, referred to as Ce2, Ce3, Ce4, and Ce5. Ce2 was taken just before the start of the GSST. There is a delay of approximately 20 min between the production of cortisol by the adrenal glands and the detectability of representative levels of cortisol in saliva. Ce2 hence reflects HPA-axis activity 20 min earlier, when the participants filled out a rating scale, and is considered a pretest measure. The first cortisol sample (Ce1), taken at the start of the experimental session (approximately 1 h before the start of the GSST), could not be used as pretest sample in the analyses because of relatively high cortisol levels, probably reflecting anticipation stress. Ce3 was collected directly after the end of the GSST and reflects HPA-axis responses during speech. Ce4 and Ce5, collected 20 respectively 40 min after the end of the GSST are considered measures of post-stress activity of the HPA-axis.

2.3. Measures

Cortisol was assessed from saliva by the Salivette sampling device (Sarstedt, Numbrecht, Germany) containing a small swab in a plastic tube on which the participants had to chew for 60 s, until the swab was soaked with saliva. This manner of collecting cortisol is relatively stress-free compared to collection by venipuncture (Schmidt, 1997). Correlations between saliva cortisol levels and serum cortisol concentrations are high (Kirschbaum and Hellhammer, 1994; Goodyer et al., 1996). After the experimental session, the samples were placed in a refrigerator at 4 °C, and within half a week brought to the laboratory of the University Medical Center in Groningen, where they were stored at −20 °C until analysis. The intra-assay coefficient of variation was 8.2% for concentrations of 1.5 nM, 4.1% for concentrations of 15 nM, and 5.4% for concentrations of 30 nM. The inter-assay coefficients of variation were, respectively, 12.6%, 5.6%, and 6.0%. The detection border was 0.9 nM. Missing morning cortisol samples values (Co1, $n = 28$; Co2, $n = 18$) were due to the following reasons: participants forgot to bring the tubes (47%), incorrect sampling (eating or brushing teeth between samples, or more than 35 min between the samples, 51%), or the invitation for the session did not include tubes (2%). Experimental samples were missing (Ce1, $n = 13$; Ce2, $n = 12$; Ce3, $n = 8$; Ce4, $n = 10$; Ce5, $n = 12$) due to detection failures in the lab (60%) or insufficient saliva in the tubes (40%). Missing values were imputed on the basis of group mean and standard deviation for the missing cortisol sample and the mean of participants' cortisol samples that were present.

Cortisol response variables were computed to compare the response to awakening and the social stress task with the other variables. CAR was calculated by subtracting Co1 from Co2. In order to calculate the response to the GSST we first calculated the peak cortisol production (indicated by Ce3, Ce4 or Ce5). The maximum increase (Max increase) was computed by subtracting Ce2 (pretest) from this peak.

Menstrual phase was calculated on the basis of the first day of last menses and the cycle length, which were both asked during the experimental session. Only girls with a regular cycle between 21 and 35 days were included in the analyses concerning menstrual phase. Menstrual cycle phase was classified as follicular or luteal. Of the 167 girls, 57.7% had a regular cycle of 28 days. Shorter or longer cycles are generally reflected more strongly in the follicular than in the luteal phase (Fox, 1999). With this in mind we defined the follicular phase as the period between the first day of the cycle and 14 days before the end of the cycle ($n = 83$), while the luteal phase was defined as the last 14 days of the cycle ($n = 84$).

Current use of oral contraceptives (OC) was assessed at the day of the experiment, while type and name of the pill were asked as part of a questionnaire that was assessed previously, at school. In total, OCs were used by 125 girls, of whom 78 (70.4%) used a monophasic OC (ethinylestradiol and progestin) and 8 a tri-phasic OC. Ten girls used the 'Diane' pill, which contains only progestin (cyproteronacetaat) and is mainly prescribed for the beneficial effects on Acne vulgaris. Nineteen girls did not know what type of pill they used.

Subjective arousal was assessed by means of the Self-Assessment Manikin (SAM), a non-verbal pictorial assessment technique to measure the pleasure, arousal and dominance associated with a person's affective reaction to a stimulus (Bradley and Lang, 1994). Participants could rate their arousal on a 9-point scale ranging from 1 (totally aroused) to 9 (not at all aroused). Arousal was assessed directly after the GSST.

Smoking behavior was assessed by questions on past and current smoking behavior in a questionnaire which was filled out at school, on average 3.07 months (S.D. = 5.12) before the experimental session. We distinguished between non-smokers ($n = 515$) and habitual smokers (i.e., at least 1 cigarette a day, $n = 111$).

Current depressed mood was assessed at the start of the experimental session, by means of the Dutch version of the short Profile of Mood Scale (POMS; Wald and Mellenbergh, 1990). The scale includes eight items describing current mood (down, helpless, sad, lonely, unhappy, unworthy, melancholic, desperate), which could be rated on a 5-point scale (1 = not at all, 2 = a little, 3 = partly, 4 = kind of, 5 = very much). Cronbach's alpha was .87. The total depression score was dichotomized at the 85th percentile.

2.4. Statistical analysis

Means and standard deviations of all variables used were calculated separately for boys, free-cycling girls in the follicular phase, free-cycling girls in the luteal phase, and OC using girls. To examine effects of gender, menstrual cycle phase and OC we compared three group pairs: (1) boys versus free-cycling girls, (2) free-cycling girls in the luteal phase

versus free-cycling girls in the follicular phase and (3) girls using OC versus free-cycling girls. Cortisol responses to awakening (CAR) were calculated as Co2 minus Co1; while cortisol responses to social stress (referred to as maximum increase) were calculated by subtracting the pretest level (Ce2) from the highest level during or after the test (Ce3, Ce4, or Ce5). First, differences between the four groups were examined by χ^2-tests (dichotomous variables) or ANOVAs (continuous variables). In case of a significant group difference, χ^2-test in 2×2 tables and *t*-tests were performed to examine differences within the three before-mentioned group pairs. Associations between variables were investigated by Pearson's correlation coefficients. Cortisol data were log transformed (using the natural logarithm) to approach a normal distribution before analysis. Tables and figures show non-transformed data. Within-subjects changes in cortisol levels were examined by repeated-measures General Linear Modeling (GLM). The two morning cortisol measures (Co1 and Co2) were used to examine the awakening response, while cortisol responses to the GSST were examined in a model including the measures Ce2, Ce3, Ce4, and Ce5. Significant between-subjects effects indicate differences in cortisol levels between the groups. Effects of gender, OC use, and menstrual phase on cortisol responsivity to awakening or social stress were tested by interactions of these variables with the within-subject factor. Significant within-subjects interaction effects indicate differences between the cortisol levels within a group. In case of significant within-subject changes in the cortisol levels before, during, and after the GSST, linear and quadratic trends were tested to examine the nature of the differences. A linear trend signifies a rise or fall in cortisol levels during the test, while a quadratic trend indicates a cortisol response to the GSST, that is, higher levels during the test than before or after it. When sphericity could not be assumed, the within-subject effects were corrected by the Greenhouse—Geisser procedure. Smoking, age and depressed mood can influence HPA-axis responses to awakening (Wüst et al., 2000; Stetler and Miller, 2005) and stress (Kirschbaum et al., 1993; Burke et al., 2005), and may also be related to gender, menstrual phase or OC use. We therefore included these variables as covariates in the GLM analyses. In the analyses of the cortisol response to awakening we included awakening time to correct for its presumed influence (Kudielka and Kirschbaum, 2003). The time of the day of the laboratory session (morning versus afternoon) was included as covariate in all analyses regarding the GSST. Although cortisol levels were expected to be higher in the morning than in the afternoon because of the diurnal rhythm of cortisol excretion, cortisol responses were assumed to be comparable during morning and afternoon sessions (Kudielka et al., 2004). To check whether this assumption held true for all groups examined, we compared morning and afternoon cortisol levels and responses within each of the subgroups (boys, free-cycling girls follicular phase, free-cycling girls luteal phase, OC using girls). A *p*-value $\leq .05$ was considered statistically significant.

3. Results

3.1. Descriptive statistics

Mean scores of the variables used are shown in Table 1. The means are presented separately for boys, free-cycling girls in the follicular phase (Girls F), free-cycling girls in the luteal phase (Girls L), and girls using oral contraceptives (Girls OC). We found significant group differences for smoking status, depressed mood, age, awakening time, Co1, Co2, Ce3, CAR and maximum increase to the GSST. Girls using OC were more often habitual smokers compared to free-cycling girls ($\chi^2(1, 284) = 18.57$, $p < .001$). Free-cycling girls were more depressed than boys ($F(1, 514) = 6.91$, $p = .01$). Within

Table 1 Descriptive statistics.

	Boys		Girls F		Girls L		Girls OC	
	n	Mean (S.D.)	*n*	Mean (S.D.)	*n*	Mean (S.D.)	*n*	Mean (S.D.)
Afternoon sessions	352	48.9%	83	54.2%	84	52.4%	125	48.8%
Habitual smokers	342	14.0%	81	16.0%	81	9.9%	122	34.4%
Depressed mood	348	1.21 (.37)	83	1.39 (.53)	84	1.22 (.30)	125	1.37 (.47)
Age (year)	352	16.12 (0.58)	81	15.96 (0.49)	83	16.05 (0.55)	124	16.36 (0.63)
Awakening (h:min)	313	7:39 (1:08)	78	7:27 (1:05)	78	7:37 (1:15)	117	7:51 (1:14)
Subjective arousal	350	5.83 (1.93)	82	5.77 (1.81)	84	6.13 (1.50)	125	5.74 (2.10)
Co1 (nmol/l)	308	7.51 (4.16)	75	8.69 (5.00)	77	8.54 (5.61)	108	8.68 (9.20)
Co2 (nmol/l)	308	12.09 (5.91)	75	15.07 (7.25)	77	14.74 (5.76)	108	13.52 (13.11)
Ce1 (nmol/l)	349	5.01 (3.58)	83	5.28 (7.00)	84	4.62 (3.09)	125	6.58 (4.71)
Ce2 (nmol/l)	349	3.73 (2.72)	83	3.55 (7.52)	84	3.47 (3.28)	125	5.11 (5.02)
Ce3 (nmol/l)	349	5.20 (3.16)	83	4.30 (7.48)	84	4.08 (3.03)	125	4.59 (3.87)
Ce4 (nmol/l)	349	4.83 (3.48)	83	4.35 (6.39)	84	4.68 (4.60)	125	4.14 (2.90)
Ce5 (nmol/l)	349	3.90 (3.11)	83	3.92 (4.93)	84	3.92 (3.90)	125	3.94 (2.96)
CAR	308	5.39 (5.10)	75	6.37 (5.98)	77	6.20 (6.73)	108	4.85 (5.67)
Max increase	349	1.92 (3.13)	83	1.57 (3.01)	84	1.78 (3.88)	125	-0.19 (2.23)

Note: Girls F = free-cycling girls in the follicular phase; Girls L = free-cycling girls in the luteal phase; Girls OC = girls using oral contraceptives; Co1 = cortisol directly after awakening; Co2 = cortisol 30 min after awakening; Ce2 = pre-stress cortisol; Ce3 = cortisol during social stress; Ce4 = post-stress cortisol; Ce5 = post-stress cortisol; CAR = cortisol awakening reponse; max increase = maximum increase in cortisol with respect to the prestress cortisol (Ce2) during the GSST.

Table 2 Bivariate associations between the variables used in this study.

	Time	Smo	Dep	Age	Sub	Awak	Co1	Co2	Ce1	Ce2	Ce3	Ce4	Ce5	CAR
1. Session time														
2. Smoking behavior	−.02													
3. Depressed mood	**.11**	−.05												
4. Age	**−.09**	.07	.01											
5. Subjective arousal	**.21**	**.09**	.02	**.10**										
6. Awakening time	−.01	.06	**−.16**	−.03	.04									
7. Co1	.03	−.05	−.01	−.01	.05	−.03								
8. Co2	.01	−.05	−.04	.05	**−.10**	−.05	**.71**							
9. Ce1	**−.23**	**.16**	−.01	**.10**	.02	−.02	**.15**	**.13**						
10. Ce2	**−.20**	**.13**	.05	**.11**	.02	−.02	**.19**	**.16**	**.76**					
11. Ce3	**−.19**	.02	**.10**	.07	−.01	−.07	.06	.06	**.57**	**.77**				
12. Ce4	**−.13**	.03	.06	.03	.00	**−.09**	**.13**	**.11**	**.56**	**.71**	**.89**			
13. Ce5	**−.14**	.07	.06	.06	.03	**−.09**	**.24**	**.18**	**.62**	**.76**	**.79**	**.90**		
14. CAR	−.01	−.02	−.05	.07	**−.19**	−.04	−.01	**.69**	.03	.03	.02	.02	.02	
15. Max increase	.05	**−.14**	.08	**−.09**	−.04	**−.09**	**−.12**	**−.11**	**−.19**	**−.27**	**.35**	**.45**	**.24**	−.03

Note: Bold values = significant association at $p < .05$; Co1 = cortisol directly after awakening; Co2 = cortisol 30 min after awakening; Ce2 = pre-stress cortisol; Ce3 = cortisol during social stress; Ce4 = post-stress cortisol; Ce5 = post-stress cortisol; CAR = cortisol awakening reponse; max increase = maximum increase in cortisol with respect to the prestress cortisol (Ce2) during the GSST.

free-cycling girls, girls in the luteal phase were more depressed than girls in the follicular phase ($F(1, 166) = 6.21$, $p = .01$). Furthermore, boys were a bit older than free-cycling girls ($F(1, 514) = 4.42$, $p = .04$) and girls using OC were older than free-cycling girls ($F(1, 287) = 26.57$, $p < .001$). Girls using OC woke up somewhat later than free-cycling girls ($F(1, 272) = 4.41$, $p = .04$). The groups did not differ with respect to the amount of subjective arousal reported. Group differences regarding any of the cortisol variables will not be discussed here, because they were tested in the GLM analyses, accounting for relevant covariates. Bivariate associations are given in Table 2. As expected, the time of the experimental session was associated with cortisol levels during that session. Furthermore, it is remarkable that most cortisol variables were associated with each other, but the CAR was neither related to any of the cortisol variables during the experimental session, nor to the (maximum) cortisol response during that session. Fig. 1 shows cortisol levels after awakening and during the morning and afternoon sessions of the experimental session. The first cortisol sample, taken approximately 1 h before the GSST, is also displayed in this figure. Among other things, Fig. 1

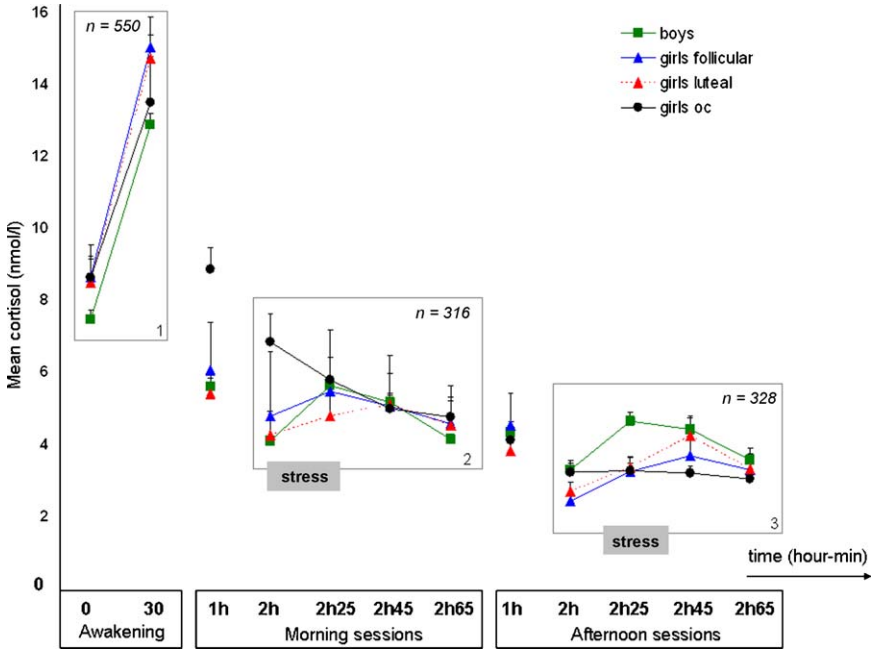

Figure 1 Mean salivary cortisol responses to awakening and social stress. *Note:* Bars represent standard errors of the mean (S.E.M.). Block 1: cortisol response to awakening; Block 2: cortisol response to the Groningen Social Stress Task during morning sessions; Block 3: cortisol response to the Groningen Social Stress Task during afternoon sessions.

Table 3 Main effects of gender, menstrual cycle phase and oral contraceptive use on cortisol and interactions with the cortisol awakening response.

Variable	*F*-statistics	*p*-Value
Gender	$F(1, 425) = 7.32$	.01
Gender × CAR	$F(1, 425) = 1.13$	.29
Oral contraceptive use	$F(1, 244) = 1.10$	.30
OC × CAR	$F(1, 244) = 5.30$	.02
Menstrual cycle phase	$F(1, 137) = 0.45$	.50
Phase × CAR	$F(1, 137) = 0.11$	.74

Note: Gender = boys vs. free-cycling girls; OC = girls using oral contraceptives vs. free-cycling girls; menstrual cycle phase = girls in the follicular phase vs. girls in the luteal phase. All analyses were corrected for depressed mood, smoking status, age and time of awakening. Adjusted degrees of freedom are reported.

shows that girls using OC showed no cortisol response towards the social stress test and had high pretest levels in the morning.

3.2. Cortisol response to awakening

Effects of gender, OC and menstrual phase on the cortisol response to awakening are presented in Table 3 and graphically represented in Fig. 1. Boys had lower morning cortisol levels than girls, but boys and girls did not differ in their response to awakening. OC users and free-cycling girls did not differ in their morning cortisol levels, but OC using girls showed a blunted response to awakening. We did not find any effect of menstrual cycle phase.

3.3. Cortisol response to the GSST

The results of the effects of gender, OC use and menstrual cycle phase on the cortisol response to social stress are presented in Table 4. Boys and free-cycling girls differed with respect to the overall cortisol levels during the GSST. Furthermore, we observed a significant interaction of gender with the cortisol response, particularly due to differences in the quadratic trend, indicating that boys reacted differently to the social stress test than free-cycling girls. As Fig. 1 shows, cortisol responses were stronger in boys than in girls. Cortisol levels and responses were also modified by OC use. Differences between OC users and free-cycling girls pertained to both the linear and the quadratic trend in the cortisol response. As opposed to free-cycling girls, cortisol levels of OC users linearly decreased during the GSST and no response was displayed whatsoever, not in the morning and not in the afternoon sessions. Girls in the luteal phase did not differ from girls in the follicular phase regarding cortisol levels or responses towards the GSST.

3.4. Effect of time of day

For each group we checked whether the cortisol response to the GSST was comparable during the morning and afternoon session. For boys, the overall level of cortisol was higher in the morning ($F(332, 1) = 24.72$, $p < .001$) but the cortisol response to the stress task did not differ between morning and afternoon sessions ($F(3, 332) = 0.25$, $p = .77$). Girls in the follicular phase neither differ in overall cortisol levels ($F(1, 76) = 1.53$, $p = .22$) nor in their cortisol response ($F(1, 76) = 1.12$, $p = .33$) between morning and afternoon. A similar pattern was present for girls in the luteal phase (overall levels $F(1, 76) = 2.94$, $p = .09$; response $F(3, 76) = 0.48$, $p = .70$). Girls using OC had higher cortisol levels in the morning than in the afternoon ($F(1, 117) = 29.06$, $p < .001$). In addition, we found a significant interaction of OC use and time of day ($F(3, 117) = 12.57$, $p < .001$) which is only due to a difference in the linear trend ($F(3, 117) = 22.70$, $p < .001$). This is illustrated in Fig. 1, where cortisol levels of OC users in the morning decline more than they do in the afternoon. These findings indicate that although cortisol levels were higher during the morning sessions for some groups, responses to the social stress test were comparable between morning and afternoon sessions.

Table 4 Main effects of gender, menstrual cycle phase and oral contraceptives on cortisol and interactions with the cortisol response to social stress.

Variable	*F*-statistics	*p*-Value
Gender	$F(1, 494) = 13.27$	<.001
Gender × Stress	$F(3, 494) = 9.73$	<.001
Linear contrast	$F(1, 495) = 2.50$	.12
Quadratic contrast	$F(1, 495) = 16.30$	<.001
Oral contraceptive use	$F(1, 279) = 4.94$	.03
OC × Stress	$F(3, 279) = 15.12$	<.001
Linear contrast	$F(1, 279) = 19.03$	<.001
Quadratic contrast	$F(1, 279) = 14.15$	<.001
Menstrual cycle phase	$F(1, 157) = 3.49$	.06
Phase × Stress	$F(3, 157) = 0.57$	.55

Note: Gender = boys vs. free-cycling girls; OC = girls using oral contraceptives vs. free-cycling girls; menstrual cycle phase = girls in the follicular phase vs. girls in the luteal phase. All analyses were corrected for depressed mood, smoking status, age and time of day. Adjusted degrees of freedom are reported.

4. Discussion

The aim of this study was to examine salivary cortisol responses to awakening and social stress in a large sample of adolescent boys and girls. We examined moderation of cortisol responses by gender, menstrual cycle phase and OC use. With respect to the cortisol awakening response, we did not find any differences regarding gender and menstrual phase, but a slightly blunted response in girls using OC. Cortisol responses to social stress were different for boys and free-cycling girls. Unexpectedly, OC users showed no response at all but displayed linearly decreasing levels of cortisol during the social stress test. We found no effect of menstrual cycle phase on cortisol responses to social stress. Despite the differences in cortisol responses to social stress between our groups, no differences in subjectively experienced arousal were reported, which is in concordance with Kirschbaum et al.'s (1999) study in adults. Consistent with findings reported by Kudielka et al. (2004), free saliva cortisol levels were higher in the morning than in the afternoon due

to the circadian rhythm of cortisol, but cortisol responses to the social stress task appeared similar at both times of the day, at least for boys and free-cycling girls.

The findings should be interpreted in the light of the following strengths and limitations. To the best of our knowledge, we are the first to study the effects of gender, OC use and menstrual cycle on cortisol responses in a non-clinical adolescent population. Our sample size is extraordinarily large (>500) for this kind of research, and provides a relatively large power to detect differences and prevent false–negative results. The fact that we examined cortisol responses to awakening as well as to psychological stress allowed us to observe differences between various kinds of HPA-axis activation. An additional strength of this study, compared to most other ones, is the relative short period of OC use in this age group. When OC are used for longer time periods, it is harder to distinguish between immediate effects of the OC and effects due to changes in physiological set points. Despite the large sample size, we were not able to study differences regarding type of OC because only 10 girls used an OC only based on progestin, which can be considered a limitation of the study. Another limitation is that menstrual cycle phase was determined via self-report and not via more objective measures, such as serum levels of estradiol and progesterone. Furthermore, since we did not asses ACTH, serum cortisol, or markers of the sympathetic nervous system, we cannot conclude that the physiological stress response was absent in OC users, but only that it was not detectable in salivary cortisol levels.

We found no effects of gender on the cortisol awakening response, which is in concordance with findings of Edwards et al. (2001) and Kudielka and Kirschbaum (2003). It should be noted, however, that women may show a sustained CAR after 30 min post-awakening (Pruessner et al., 1997; Wüst et al., 2000). Since we only examined cortisol levels during the first 30 min after awakening, we could not study this possible gender difference. We did find an effect of use of OC: the CAR was blunted in girls using OC compared to free-cycling girls. Pruessner et al. (1997) also reported an attenuated CAR in adult subjects using OC ($p = .10$, $n = 35$) but Wüst et al. (2000) found no effects of OC use. In addition to a blunted CAR, we found high pretest cortisol values in the morning session in girls using OC, which suggests a slower increase and decrease of cortisol levels after awakening. In concordance with Kudielka and Kirschbaum (2003), we found no differences in the CAR according to menstrual cycle phase.

With respect to responses to social stress, boys displayed a stronger response to the social stress test than girls which is consistent with the gender difference in cortisol responses to social stress often reported in adult populations (Kudielka and Kirschbaum, 2005). It is further interesting to note that boys had the highest cortisol levels at the beginning of the social stress task, while girls had the highest levels at the end. This delayed peak in girls could be due to a slower activation of the HPA-axis or may be caused by the social stress task. Possibly, the speech task (talking about yourself) was experienced as more stressful by the boys, while the mental arithmetic task was more stressful for girls.

With regard to menstrual cycle phase, we found no significant effect of menstrual cycle phase on adolescents' cortisol responses which is contrary to previously reported findings in adults (Altemus et al., 1997; Kirschbaum et al., 1999; Wolf et al., 2001; Rohleder et al., 2003). But we do see a similar pattern as observed in adults, that adolescent girls in the follicular phase tended to have lower cortisol levels than girls in the luteal phase. This could be due to the higher levels of estrogen in the follicular phase which increase the amount of CBG-bound cortisol, leaving less free cortisol for detection in saliva. A possible reason why we did not find a significant effect of phase on cortisol responses to stress might be that the adolescent menstrual cycle is not as stable as the cycle of adult women. Lower levels or a different ratio of sex hormones in adolescent girls, as compared to adult women, could influenced the response of the HPA-axis to stress in a different manner.

Prior studies in adult subjects revealed blunted responses to a social stress task in OC users, while we found no response at all in our adolescent population. It is possible that the total lack of cortisol responses in our OC using girls, as opposed to the blunted (but still present) responses reported previously in adults, is due to lower stressfulness of the GSST compared to the original Trier Social Stress Test (TSST), which involves a life jury instead of a single experimenter and a video camera. In fact, the mean cortisol response reported in this study were lower than those reported by Kirschbaum et al. (1993), which may indicate that the GSST was less stressful indeed.

Despite the fact that the GSST may have been less stressful than the TSST, it is unlikely that OC using girls were not at all stressed by our social stress task, since they reported to be equally stressed as boys and free-cycling girls. The lack of response in the OC users during the morning sessions could be explained by the high pretest levels of cortisol which prevents further increase. Kirschbaum et al. (1999) showed by means of a 12-h salivary free cortisol profile that adult OC users had also higher cortisol levels between 9 and 11 a.m. than men and free-cycling women. However, the high pretest levels in the morning found in this study cannot explain the absence of a cortisol response in the afternoon sessions, when pretest levels were comparable to those of boys and free-cycling girls.

With respect to adolescent OC users, the results from this study should be interpreted with caution. The absence of a salivary cortisol response does not implicate that there was no physiological response to stress whatsoever. The CRF (corticoid releasing factor) signal from the hypothalamus did not result in increases in saliva cortisol for some reason, but this signal can still activate the sympathetic nervous system, resulting for example, in increased heart rate. The effects of synthetic hormones in orally taken contraceptives on the activation and feedback of the HPA-axis are difficult to predict, since they can act on several tissues and within different time domains. In adult women it has been shown that OC use led to increased CBG levels, and for this reason, smaller fractions of free cortisol could be detected in saliva (Kumsta et al., 2007). However, the high levels of cortisol in the morning indicate that this mechanism may not be that prevalent in adolescent girls using OC. The discrepancy in findings between adult women and adolescent girls might be related to the duration period of OC use and the time to adjust to the effects of OC intake. Longitudinal research is needed to elucidate the effects of oral contraceptive use on HPA-axis functioning in adolescence, since especially long-term effects of OC use are still unknown (Ott et al., 2008).

To conclude, the HPA-axis is one of the most important stress systems and dysfunctions may result in maladaptive responses and failure to cope with stress. Inappropriate coping will, in turn, interfere with a healthy psychosocial development. Altered functioning of the HPA-axis can influence, for instance, levels of inflammatory cytokines, heart rate, and cognition (Owens and Nemeroff, 1992; Belanoff et al., 2001) with possible consequences for psychological and physical health. We found both similarities and differences in responses to stress in adolescents when compared to adults. It is necessary that our results are replicated in another sample of adolescents to investigate whether the new and unexpected findings are substantial or might have been chance findings.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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